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Inheritance of selected pathotoxin resistance in maize plants regenerated from cell cultures

(cell culture selection/plant regeneration/disease resistance/*Helminthosporium maydis* race T/cytoplasmic inheritance)

B. G. GENGENBACH, C. E. GREEN, AND C. M. DONOVAN

Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, Minnesota 55108

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ABSTRACT Texas male-sterile cytoplasm (cms-T) maize is susceptible to *Helminthosporium maydis* race T and its pathotoxin, whereas nonsterile cytoplasm maize is resistant. Callus cultures initiated from immature embryos of a *cms*-T genotype, $BC_1A188(T)$, were susceptible to the toxin and were capable of plant regeneration. Toxin-resistant cell lines were selected by a sublethal enrichment procedure in which cms-T callus was grown for several selection cycles (subculture transfers) in the presence of progressively higher concentrations of toxin. Periodically during the selection process, plants were regenerated from the cms-T cultures to determine their susceptibility or resistance to the toxin. Plants regenerated after four cycles of selection were male-sterile and toxin-susceptible as shown by leaf bioassays. All plants regenerated from cell lines isolated from the fifth selection cycle onward, however, were toxin-resistant and 52 of 65 were fully male-fertile. The remaining 13 "male-sterile" resistant plants did not shed pollen and did not resemble cms-T plants in tassel morphology. Some "male-sterile" plants produced anthers containing a small amount of starch-filled pollen, suggesting that the sterility of these 13 plants was not the result of the *cms*-T trait. Leaf bioassays on F_1 progeny from regenerated resistant plants indicated that resistance to the toxin was inherited only through the female. The male-fertility trait also was inherited only through the female. After inoculation with *H. maydis* race T spores, leaf lesion size for progeny from regenerated resistant plants coincided with their reaction to the toxin. This result indicated that plant resistance to the pathogen was closely correlated with the toxin resistance obtained through cell culture selection.

Since the early demonstrations that plants could be regenerated from cultured cells of a few species—e.g., tobacco (*Nicotiana tobacum* L.) and carrot (*Doucus corota* L.)—plant geneticists have become increasingly interested in exploiting tissue culture methods to manipulate the plant genome at the cellular level and in recovering and studying these modifications in plants. Cellular selection and subsequent genetic study have been accomplished for only a few plant traits at the present time. Carlson (1) selected tobacco cultures resistant to methionine sulfoximine, a compound with effects similar to those of the pathotoxin from *Pseudomonas tabaci*, causal agent of tobacco wildfire disease. Plants regenerated from three methionine sulfoximine-resistant cell lines did not develop characteristic chlorotic halos after application of methionine sulfoximine or *P. tabaci* to their leaves. Segregation data indicated that methionine sulfoximine resistance was transmitted to F_2 progeny of regenerated plants. Carrot plants have been regenerated from cell lines resistant to 5-methyltryptophan (2). These plants possessed resistance to the analogue as evidenced by the ability of callus initiated from them to grow in the presence of 5-methyltryptophan. Plants have been regenerated from four auxotrophic tobacco cell lines requiring amino acids, vitamins, or purines for normal growth (3). The mutant traits of three plants were inherited as single gene recessives; the fourth exhibited a more complex inheritance pattern. The inheritance of 5-bromodeoxyuridine resistance in tobacco plants regenerated from resistant cell lines also has been attributed to nuclear gene(s) (4). Resistance to streptomycin also has been selected in tobacco callus (5). Regenerated plants were resistant to streptomycin, and inheritance studies of one plant indicated that resistance was transmitted to the F_1, F_2, and backcross progeny only through the female parent (6). Thus, only in tobacco have inheritance patterns been studied in plants for mutant traits selected at the cellular level.

The pathotoxin produced by *Helminthosporium maydis* (Nisikado and Miyake) race T has been used previously to select toxin-resistant cell lines from Texas male-sterile (*cms*-T) maize (*Zea mays* L.) callus (7). This pathotoxin is the disease determinant of southern corn leaf blight. In the field, southern corn leaf blight seriously damages *cms*-T plants while nonsterile cytoplasm (N) counterparts are significantly less affected under similar conditions (8–10). Growth of *cms*-T cell lines selected for toxin resistance was not inhibited by toxin levels lethal to unselected *cms*-T callus (7). This resistance has been retained for 30 months in the absence of further selection. In addition, mitochondria from the toxin-resistant *cms*-T cell lines were insensitive to the toxin, as were mitochondria from N callus. These observations suggest that a stable genetic change was selected. The inability to regenerate plants from these cultures, however, made it impossible to examine the expression and inheritance of toxin resistance in plants.

This paper discusses the selection of cell lines resistant to *H. maydis* race T pathotoxin from *cms*-T maize callus, the regeneration of plants from these resistant cell lines, and the expression and inheritance of toxin resistance in regenerated plants and their progeny.

MATERIALS AND METHODS

Callus Initiation and Plant Regeneration. The *cms*-T callus was obtained from the genotype W[9(*cms*-T)/W22 × A188 after one backcross as the female parent to A188 and is hereafter denoted as $BC_1A188(T)$. Immature embryos, 1–2 mm long, were isolated 12 days after the backcross pollination and used to initiate callus according to the procedure of Green and Phillips (11). Murashige and Skoog medium containing 1.25 mg of 2,4-dichlorophenoxyacetic acid (2,4-D) per liter, 1 mM L-asparagine, and 0.7% agar was used for initiation and maintenance of the callus cultures. Plants were regenerated from these cultures as needed by removal of the 2,4-D from the growth

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Abbreviations: *cms*-T, Texas male-sterile cytoplasm; N, nonsterile cytoplasm; 2,4-D, 2,4-dichlorophenoxyacetic acid; *rf*, nuclear allele that does not restore fertility to *cms*-T.

medium (11). Regenerated plants were transferred to a vermiculite/soil mixture and grown for 2 weeks at 27°, 70–80% relative humidity, and a 16-hr photoperiod with 1500 lux cool-white fluorescent light (12). These plants were grown to maturity in soil in 20-liter pots, either outside in the summer or in the greenhouse during the remainder of the year. In the greenhouse the available natural light was supplemented with a 15-hr photoperiod of approximately 3000 lux provided by 400-W Lucalux, high-pressure sodium lamps (General Electric Co., Cleveland, OH).

Selection Procedure. Toxin obtained from shake cultures of *H. maydis* race T was prepared as described (7). Before use, it was concentrated 20-fold compared to the initial filtrate. Aliquots of toxin were added to the medium before autoclaving to give final concentrations of 0.05–2.5% (vol/vol). During the initial selection cycle the *cms*-T callus was cultured on medium containing low to moderately inhibitory toxin concentrations (0.05–0.25%). In each subsequent selection cycle (one subculture transfer), callus inocula were placed on media with either the same or a greater toxin concentration. The callus inocula used in each new selection cycle were chosen visually based on their ability to grow at the previous toxin level and on an appearance suggesting that the callus would differentiate if placed on regeneration medium. Transfers were made at 4- to 7-week intervals. Callus stocks were increased for five transfers before selection was initiated and four selection cycles were completed before the first plants were regenerated. Regeneration medium did not contain toxin.

Characterization of Regenerated Plants. The reaction of regenerated plants to the toxin was determined on leaves in *situ*. A 3-mm-diameter hole was made adjacent to the midrib and a 1-cm^2 filter paper was rolled into a cylinder and inserted into the hole. Then 50 μl of full-strength toxin was applied to the filter paper. Tests of this toxin bioassay procedure showed that *cms*-T leaves developed long chlorotic lesions whereas N leaves showed no reaction. Control solutions of water or uninoculated fungal culture medium gave no reaction when applied to either *cms*-T or N leaves. Each regenerated plant also was classified for male sterility or fertility and other morphological features.

Male-sterile regenerated plants were crossed with the inbred line A188N*rf* (nonrestorer of fertility). Male-fertile plants were self-pollinated if possible or were crossed by A188N*rf*. Fertile plants also were crossed onto either Wf9(*cms*-T) or A619(*cms*-T) × A188 plants. Progeny obtained from crosses with regenerated plants were grown for 4–5 weeks in a greenhouse sandbench and watered with Hoagland's solution. The reaction of the progeny to the toxin was determined by the bioassay procedure described above for regenerated plants. Reaction of some progeny to infection by *H. maydis* race T was determined by spraying a spore suspension (1000 spores per ml) on the fifth and sixth leaves and covering the sandbench with plastic to raise the humidity and temperature for 24 hr. Average lesion length was determined 10 days after infection. Plants from some crosses also were transferred from the sandbench to 20-liter pots and grown to maturity to determine male fertility or sterility.

Mitochondria Analyses. Mitochondria were isolated from callus and etiolated seedling shoots (13) and the effects of toxin on several mitochondrial activities were determined according to the methods of Gengenbach and Green (7).

RESULTS

Selection for Toxin-Resistaot Callus. Growth of callus in the initial selection cycle was inhibited at toxin concentrations of 0.05–0.25% (vol/vol). By visual estimation it was apparent that growth was inversely related to toxin concentration; most callus inocula used for the second selection cycle were obtained from cultures grown on 0.05% and 0.1% toxin. Modest selection pressure was used initially because the effect of severe toxin inhibition on the ability to regenerate plants was not known. Earlier observations indicated that immediate selection at high toxin levels might destroy the capacity of resistant cell lines to regenerate plants.

Table 1. Phenotype of plants regenerated from *cms*-T callus during selection for toxin resistance

Selection cycle	Toxin conc., %	Cell line		Regenerated plant phenotype, no. of plants*		
				Male-sterile susceptible	"Male-sterile" resistant	Male-fertile resistant
4	0.05	—		4	0	0
4	0.10	—		5	0	0
			Totals	9	0	0
5	0.10	—		0	0	2
6	0.25	—		0	0	2
6	0.25	R2		0	0	3
7	0.25	—		0	3	3
7	0.25	R1		0	1	0
7	0.50	R1		0	1	0
7	0.50	R2		0	0	9
10	0.50	—		0	1	6
10	1.0	R5		0	0	1
10	1.0	R6		0	0	1
10	1.0	R7		0	0	1
10	1.0	R10		0	0	2
10	1.0	R14		0	0	1
10	1.0	R16		0	0	1
10	2.0	—		0	0	1
10	2.0	R2		0	1	12
11	1.0	R2		0	1	0
11	1.0	R6		0	1	1
11	1.0	R17		0	2	1
11	2.0	R2		0	0	3
12	1.0	R1		0	2	1
12	1.0	R6		0	0	1
			Totals	0	13	59

* The "male-sterile" phenotype represents plants that did not shed pollen because of abnormal tassel, anther, or pollen development.

Phenotypes of Regenerated Plants. After four selection cycles, nine plants were regenerated from callus and grown to maturity (Table 1). These plants developed large chlorotic lesions, indicating susceptibility, when toxin was applied to their leaves (Fig. 1). They also were male-sterile. Thus, the phenotype of these plants coincided with that of the parental genotype. After the fifth selection cycle, two plants were regenerated that gave resistant reactions to the toxin bioassay (Fig. 1). These plants were male-fertile. This dramatic change in phenotype was surprising because no clearly superior resistant cell lines had been identified at that time.

The first obvious visual evidence of toxin resistance at the callus level was obtained in the sixth cycle: two cell lines, R1 and R2, grew much faster on 0.25% toxin than did other inocula in the same flasks (Fig. 2). These cell lines, and several others recovered in later selection cycles (Table 1), were maintained on medium containing toxin at concentrations up to 2%. In all, 65 plants have been regenerated from these resistant cell lines, grown to maturity, and tested for susceptibility or resistance

Table 3. Free amino acids in heterozygous (*Ltr**-19/+) and homozygous (*Ltr**-19/*Ltr**-19) LT-resistant and -susceptible (+/+) seed†

Amino acid‡	Genotypes,§ μmol/g dry weight			*Ltr**-19/*Ltr**-19 to +/+ ratio
	+/+	*Ltr**-19/+	*Ltr**-19/*Ltr**-19	
Threonine	0.15 ± 0.01	4.35 ± 0.35	11.15 ± 1.33	76.8
Serine	0.74 ± 0.10	0.81 ± 0.10	2.37 ± 0.25	3.2
Proline	0.35 ± 0.09	2.63 ± 0.80	1.39 ± 0.19	4.0
Methionine	0.10 ± 0.03	0.05 ± 0.01	0.36 ± 0.09	3.7
Lysine	0.15 ± 0.04	0.16 ± 0.03	0.19 ± 0.01	1.3
Arginine	Trace	Trace	Trace	—
Total	6.74	12.08	19.70	
Total kernel	0.84	1.62	2.54	

† Each value represents the mean ± SEM of four replications.
‡ Genotype had little affect on the level of other amino acids assayed: aspartic acid, 1.1–1.7 μmol/g dry weight; glutamic acid, 1.0–1.6; glycine, 0.23–0.35; alanine, 0.35–0.63; valine, 0.35–0.49; isoleucine 0.10–0.19; leucine, 0.07–0.08; tyrosine, 0.18–0.21; histidine, 0.16–0.27.
§ *Ltr**-19/*Ltr**-19 seed was obtained by self-pollination of a third generation *Ltr**-19/*Ltr**-19 plant, which was also crossed to +/+ to produce the *Ltr**-19/+ seed; +/+ seed was from an unselected regenerated control plant crossed to A188 and then backcrossed to A188 in the second generation.

The effect of threonine overproduction in *Ltr**-19/+ and *Ltr**-19/*Ltr**-19 on total kernel threonine is assessed in Table 4. These data show that the mutant kernels are at least as large as normal and that the protein content in kernels of all genotypes was not greatly diffferent. The threonine content of the kernel proteins also was very near 4% in all genotypes. The large increase in free threonine in *Ltr**-19/+ and *Ltr*-19/*Ltr**-19 kernels contributed to substantial increases in total threonine. The elevated free threonine present in *Ltr**-19/*Ltr**-19 increased the total threonine in these kernels by 33–59%.

DISCUSSION

Our data clearly indicate a mutational origin for LT resistance in the LT19 line. Resistance is expressed both in tissue cultures and seedlings of progeny from plants carrying *Ltr**-19. Pollen transmission of LT resistance indicates that the trait is coded by a nuclear gene. χ^2 analysis of progeny from cross-pollinations showed that the segregation of LT resistance was not significantly different from a single dominant gene (Table 1). However, segregation in self-pollinations of *Ltr**-19 plants did deviate significantly from the expected 3:1 resistant/susceptible frequency. The reduced frequency could be accounted for by reduced transmission of gametes carrying the *Ltr**-19 gene. Data from reciprocal crosses showed that when *Ltr**-19/+ plants were used as male parents. LT-carrying pollen was reduced in competitive effectiveness. Cytological analysis of meiosis in LT-resistant plants has shown the probable presence of a duplication that could account for the reduced transmission of LT resistance in certain crosses if the duplication and the *Ltr**-19 gene were linked.

The LT selection procedure in this study was modified from previous work (12) to include the use of recently initiated cultures for mutant selection and the regeneration of plants from resistant cultures as soon as possible after they were identified. Selection was completed in 3 months instead of 6 and was carried out at a higher concentration of LT. Sodium azide also was applied to the cultures to increase possibly the mutation frequency and, thus, the likelihood of obtaining LT-resistant cultures. Because only one mutant was obtained, no conclusions can be made about the effectiveness of azide as a mutagen in maize tissue cultures.

LT resistance has not resulted in any immediately visible changes in the plants during development and maturation in the greenhouse or field. Plants carrying the *Ltr**-19 gene appear normal and as vigorous as comparable control plants. Because LT resistance is strongly expressed in the roots (Fig. 2*B*), it can be detected routinely with the seedling root bioassay. Shoot development appears less responsive to LT inhibition (Fig. 2*A*), and consequently the expression of LT resistance in this organ is less clear. It is not known whether LT directly inhibits amino acid synthesis and growth in shoots or whether shoots are affected indirectly through the effect of LT on roots.

The increased concentration of free threonine in kernels carrying *Ltr**-19 (Table 3) corresponds to the increased free threonine observed in LT-resistant tissue cultures (Table 2). The magnitude of the increase indicated the possibility that total threonine content of the kernel might be increased. This is borne out by the data in Table 4, which show that total threonine in *Ltr**-19/*Ltr**-19 kernels was increased by 33–59%. This ta-

Table 4. Effect of free threonine overproduction in various genotypes on total threonine in seed†

Analysis	+/+		*Ltr**-19/+	*Ltr**-19/*Ltr**-19	
	1	2	1	1	2
Kernel weight	116 ± 5	123 ± 9	154 ± 14	122 ± 9	134 ± 10
Protein/100 mg of meal	9.4 ± 0.15	10.1 ± 0.18	8.9 ± 0.11	10.4 ± 0.14	10.2 ± 0.09
Threonine/100 mg of protein	4.05 ± 0.49	3.95 ± 0.41	3.81 ± 0.29	3.99 ± 0.42	3.90 ± 0.44
Protein threonine/kernel	0.44 ± 0.05	0.49 ± 0.05	0.52 ± 0.06	0.51 ± 0.06	0.53 ± 0.04
Free threonine/kernel	<0.01 ± <<0.01	<0.01 ± <<0.01	0.07 ± 0.01	0.17 ± 0.02	0.31 ± 0.03
Total threonine/kernel	0.44	0.50	0.59	0.68	0.84
Free threonine as % of protein threonine	0.4%	0.6%	13%	33%	59%

† Each value (in mg except last item) represents the mean ± SEM of three replications.

ble also indicates that kernel weight, total protein, and threonine content of protein were not altered appreciably between the +/+, *Ltr**-19/+, and *Ltr**-19/*Ltr**-19 genotypes.

In conclusion, a mutation, *Ltr**-19, has been isolated from tissue culture that conditions the overproduction of a nutritionally essential amino acid in tissue cultures and, most importantly, in the seed of regenerated plants and subsequent generations. The magnitude of threonine overproduction in seed carrying *Ltr**-19 offers the opportunity to isolate and use amino acid-overproducer mutants to increase selectively the levels of specific nutritionally limiting amino acids in the seeds of crops.

Nutritionally, threonine is the third most limiting of the essential amino acids in maize, but in several other cereals, particularly wheat and rice, it is the second limiting amino acid (19). The more serious threonine deficiency in these species, coupled with the results of our study, demonstrate the need to initiate selections for LT resistance. The results of our study also clearly indicate the desirability of isolating lysine-, tryptophan-, and methionine-overproducer mutants in various crops.

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1. Carlson, P. S. (1973) Science 180, 1366–1368.
2. Maliga, P., Sz-Breznovits, A. & Marton, L. (1973) Nature (London) 255, 401–402.
3. Yurina, N. P., Odintsova, M. S. & Maliga, P. (1978) Theor. Appl. Genet. 52, 125–128.
4. Gengenbach, B. G., Green, C. E. & Donovan, C. M. (1977) Proc. Natl. Acad. Sci. USA 74, 5113–5117.
5. Chaleff, R. S. & Parsons, M. F. (1978) Proc. Natl. Acad. Sci. USA 75, 5104–5107.
6. Bourgin, J. P. (1978) Mol. Gen. Genet. 161, 225–230.
7. Green, C. E. & Phillips, R. L. (1974) Crop Sci. 14, 827–830.
8. Dunham, V. L. & Bryan, J. K. (1971) Plant Physiol. 47, 91–97.
9. Green, C. E. & Donovan, C. M. (1980) Crop Sci. 20, 358–362.
10. Bryan, J. K. (1969) Biochim. Biophys. Acta. 171, 205–216.
11. Bryan, P. A., Cawley, R. D., Brunner, C. E. & Bryan, J. K. (1970) Biochem. Biophys. Res. Commun. 41, 1211–1217.
12. Hibberd, K. A., Walter, T., Green, C. E. & Gengenbach, B. (1980) Planta 148, 183–187.
13. Green, C. E. & Phillips, R. L. (1975) Crop Sci. 15, 417–421.
14. Green, C. E. (1977) Hort. Sci. 12, 131–134.
15. Bielinski, R. L. & Turner, N. A. (1966) Anal. Biochem. 17, 278–293.
16. Sodek, L. & Wilson, C. M. (1971) J. Agr. Food Chem. 19, 1144–1150.
17. Nilan, R. A., Sideris, E. G., Kleinhofs, A., Sander, C. & Konzak, C. F. (1973) Mutat. Res. 17, 142–144.
18. Sarma, N. P., Patnaik, A. & Jachuck, P. J. (1979) Environ. Exp. 19, 117–121.
19. McLaughlin, J. M., Venkat, R. S., Noel, F. J. & Morrison, A. B. (1967) Can. J. Biochem. 45, 31–37.

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* The "male-sterile" phenotype represents plants that did not shed pollen because of abnormal tassel, anther, or pollen development.

Phenotypes of Regenerated Plants. After four selection cycles, nine plants were regenerated from callus and grown to maturity (Table 1). These plants developed large chlorotic lesions, indicating susceptibility, when toxin was applied to their leaves (Fig. 1). They also were male-sterile. Thus, the phenotype of these plants coincided with that of the parental genotype. After the fifth selection cycle, two plants were regenerated that gave resistant reactions to the toxin bioassay (Fig. 1). These plants were male-fertile. This dramatic change in phenotype was surprising because no clearly superior resistant cell lines had been identified at that time.

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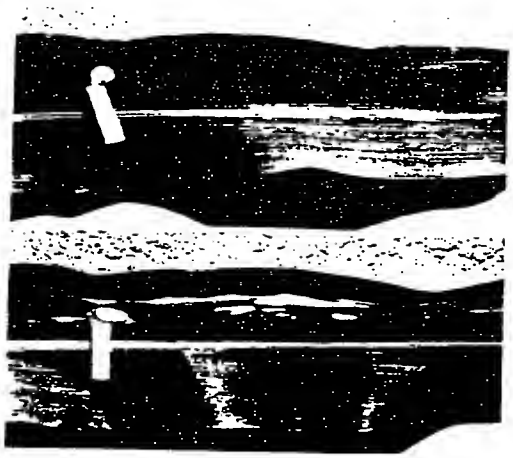

FIG. 1. Reaction to toxin in leaf bioassay. (*Upper*) Resistant reaction exhibited by A188N and all regenerated plants classified as resistant. (*Lower*) Typical susceptible reaction exhibited by BC_1A188(T) and all regenerated plants classified as susceptible.

to the toxin. All plants regenerated after the fifth selection cycle have given resistant reactions in leaf bioassays.

The second trait of special interest in the regenerated plants was male sterility. Fifty-two of the 65 resistant plants were male-fertile (Table 1). The remaining 13 "male-sterile" resistant plants generally exhibited abnormal morphological characteristics in the form of poorly developed tassels, pistillate florets in the tassel, short stature, or abnormal leaf development. Most "male-sterile" plants extruded some shriveled anthers but did not shed pollen. When the anther contents were stained with I2/KI, pollen grains ranging from empty to starch-filled were revealed. The association of abnormal morphology with "male sterility" in these 13 plants suggests that the sterility might not be caused by *cms*-T cytoplasm. Instead, it could be the developmental expression of physiological or cytogenetic disturbances occurring in the cultures during plant regeneration. No progeny were obtained in any crosses with these "male-sterile" plants used either as females or as males. Several plants regenerated from unselected control cultures after growth as callus for a period equivalent to 12 cycles of selection also exhibited similar abnormal morphology. The control *cms*-T plants were susceptible to the toxin and were male-sterile.

Inheritance Studies. Crosses of male-sterile, susceptible regenerated plants by A188N*rf* males always resulted in male-sterile, toxin-susceptible progeny (Table 2). Crosses of male-fertile, resistant regenerated plants by A188N*rf* males

FIG. 2. Toxin-resistant cell lines R1 (left flask) and R2 (right flask) growing in flasks containing two toxin-susceptible inocula.

Table 2. Phenotypes of progeny from crosses involving regenerated plants

Crosses*			Reaction to toxin in leaf bioassay, no. of F_1 plants	
Female parent	Male parent	No.	Susceptible	Resistant
Regenerated				
Susceptible, sterile	A188N	5	61†	0
Resistant, fertile	Selfed	6	0	65‡
Resistant, fertile	A188N	2	0	44§
	Regenerated:			
Wf9 (*cms*-T)	Resistant, fertile	5	102¶	0
A619 (*cms*-T) × A188N	Resistant, fertile	3	44	0

* Sterile and fertile indicate male-sterile and male-fertile plants, respectively. Each regenerated, resistant male-fertile plant was used both as male and as female in these crosses.

† All male-sterile.

‡ Twenty-six male-fertile, 6 "male-sterile", 33 not grown to maturity.

§ Thirty-eight male-fertile, 1 "male-sterile", 5 not grown to maturity.

¶ Sixty-nine male-sterile, 33 not grown to maturity.

‖ None grown to maturity.

always produced male-fertile resistant progeny. When the same male-fertile resistant plants, however, were used to pollinate *cms*-T plants, the progeny were always male-sterile and susceptible. When fertile, toxin-resistant regenerated plants were self-pollinated, the resulting progeny were all resistant, but both male-fertile and male-sterile plants were observed in several families. The sterile plants in these segregating families either had rudimentary tassels with no anthers or had normal tassels that extruded small anthers partially filled with pollen grains. When stained with IKI, these pollen grains ranged from empty to starch-filled. In none of the crosses involving *cms*-T females × resistant fertile regenerated males was the toxin resistance or male fertility transmitted to the progeny. The explanation for the appearance of male-sterile plants among progeny obtained by self-pollinating male-fertile regenerated plants is unknown.

Reaction to *H. maydis* Race T Infection. Although regenerated plants from resistant calli were completely resistant to the toxin in leaf bioassays, the reaction of their progeny to the disease organism also was of interest. The reaction was tested by applying *H. maydis* race T spores to plants under conditions favorable for infection. Ten days after spore application, the average lesion length on leaves of progeny from toxin-resistant regenerated plants was not different from that of the resistant controls, A188(N) and Wf9(N)·W22 × A188 (Table 3). These data also demonstrated that lesions on BC_1A188(T) plants and toxin-susceptible progeny of regenerated plants were significantly larger than lesions on toxin-resistant plants. Thus, selection for toxin resistance in callus cultures resulted in progeny from regenerated plants that were as resistant to the causal organism as N cytoplasm genotypes under the greenhouse test conditions.

Toxin Effects on Mitochondria. Tests on mitochondria isolated from BC_1A188(T) shoots and unselected callus revealed that several activity parameters were sensitive to 2.5% toxin in the reaction medium (Table 4). Oxidation of NADH was stimulated, malate-driven 2,6-dichlorophenolindophenol reduction rates and the ADP:O ratio were decreased, and a toxin-mediated mitochondrial swelling was observed. However,

ganellar DNAs and the specific effects of the toxin on activities of isolated mitochondria, but not isolated chloroplasts, support the idea that the extranuclear genome responsible for *cms*-T traits of maize resides in the mitochondrion.

This system for selecting at the cellular level can provide material suitable for examining how these changes occur in extranuclear inherited traits. Determining whether restriction endonuclease digestion patterns of mitochondrial DNA from resistant regenerated plants and progeny are converted from a *cms*-T pattern to an N pattern would be particularly interesting.

1. Carlson, P. S. (1973) "Methionine sulfoximine-resistant mutants of tobacco," *Science* 180, 1366–1368.
2. Widholm, J. M. (1974) "Cultured carrot cell mutants: 5-Methyltryptophan-resistance trait carried from cell to plant and back," *Plant Sci. Lett.* 3, 323–330.
3. Chaleff, R. S. & Carlson, P. S. (1974) "Somatic cell genetics of higher plants," *Annu. Rev. Genet.* 8, 267–278.
4. Marton, L. & Maliga, P. (1975) "Control of resistance in tobacco cells to 5-bromodeoxyuridine by a simple Mendelian factor," *Plant Sci. Lett.* 5, 77–81.
5. Maliga, P., Sz-Bresnovits, A. & Marton, L. (1973) "Streptomycin-resistant plants from callus culture of haploid tobacco," *Nature New Biol.* 244, 29–30.
6. Maliga, P., Sz-Bresnovits, A. & Marton, L. (1975) "Non-Mendelian streptomycin-resistant tobacco mutant with altered chloroplasts and mitochondria," *Nature* 255, 401–402.
7. Gengenbach, B. G. & Green, C. E. (1975) "Selection of T-cytoplasm maize callus cultures resistant to *Helminthosporium maydis* race T pathotoxin," *Crop Sci.* 15, 645–649.
8. Hooker, A. L., Smith, D. R., Lim, S. M. & Beckett, J. B. (1970) "Reaction of corn seedlings with male-sterile cytoplasm to *Helminthosporium maydis*," *Plant Dis. Rep.* 54, 708–712.
9. Lim, S. M., Hooker, A. L., Kinsey, J. G. & Smith, D. R. (1974) "Comparative grain yields of corn hybrids in normal and in Texas male-sterile cytoplasm (*cms*-T) infected with *Helminthosporium maydis* race T and disease components of *cms*-T corn hybrids," *Crop Sci.* 14, 190–195.
10. Hallauer, A. R. & Martinson, C. A. (1975) "Reaction of maize hybrids and inbreds to Southern corn leaf blight," *Agron. J.* 67, 497–501.
11. Green, C. E. & Phillips, R. L. (1975) "Plant regeneration from tissue cultures of maize," *Crop Sci.* 15, 417–421.
12. Green, C. E. (1977) "Prospects for crop improvement in the field of cell culture," *Hortic. Sci.* 12, 131–134.
13. Miller, R. J., Dumford, S. W., Koeppe, D. E. & Hanson, J. B. (1970) "Divalent cation stimulation of substrate oxidation by corn mitochondria," *Plant Physiol.* 45, 649–653.
14. Miller, R. J. & Koeppe, D. E. (1971) "Southern corn leaf blight: Susceptible and resistant mitochondria," *Science* 173, 67–69.
15. Levings, C. S., III. & Pring, D. R. (1976) "Restriction endonuclease analysis of mitochondrial DNA from normal and Texas cytoplasmic male-sterile maize," *Science* 193, 158–160.

Table 3. Relationship between length of *H. maydis* race T lesions from spore inoculation and toxin reaction on control plants and progeny of regenerated plants

Seed source and genotype	Lesion length, mm	Reaction to toxin in leaf bioassay
Control seed:		
$BC_1A188(T)$	22.5 ± 7.0	Susceptible
Wf9(N)/W22 × A188	3.9 ± 2.2	Resistant
A188(N)	5.7 ± 2.2	Resistant
Progeny of regenerated plants:*		
87 × A188(N)	25.4 ± 7.8	Susceptible
179 × A188(N)	5.8 ± 2.3	Resistant
182 selfed	5.2 ± 2.3	Resistant
Wf9(*cms*-T) × 182	14.7 ± 7.1	Susceptible

* Plant 87 (susceptible, male-sterile) was regenerated after four selection cycles and plants 179 and 182 (resistant, male-fertile) were regenerated after seven selection cycles (Table 1) from $BC_1A188(T)$ cultures.

when mitochondria from toxin-resistant R1 and R2 cell lines were tested, no toxin-mediated effects were observed. The lack of effect indicated that selection for toxin-resistant callus also had resulted in a change from toxin-sensitive mitochondria to toxin-insensitive mitochondria. Previous work has established that mitochondria isolated from shoots of N cytoplasm seedlings (14) and from N cytoplasm callus (7) are not sensitive to race T toxin. R1 and R2 cell lines provided 34 of the 65 resistant regenerated plants. Although not tested, it is expected that mitochondria from resistant regenerated plants and from their maternal progeny also would be insensitive to the toxin.

DISCUSSION

Regeneration of plants is essential for using tissue cultures for plant improvement. The current capability for regeneration of maize plants from cultures has been described recently (12). The second requirement for plant improvement is that traits selected in culture systems are expressed in the regenerated plants and are inheritable. The results presented here demonstrate the expression and inheritability of resistance to *H. maydis* race T toxin in plants after selection in maize callus cultures. Tobacco is the only other plant known to us in which genetic tests have been conducted on regenerated plants and their progeny after selection for specific traits in tissue culture (1, 3, 4, 6). Cell culture systems, however, now have advanced considerably for several important cereal crops such as barley (*Hordeum vulgare* L.), rice (*Oryza sativa* L.), and oats (*Avena sativa* L.). Attempts to select variants in tissue culture and to study their expression and inheritance in regenerated plants should be forthcoming in these crops.

The data presented in this paper indicate that toxin resistance was obtained by selection of a cell population with cytoplasm conditioning male fertility and resistance from an original cell population with cytoplasm conditioning male sterility and susceptibility. Most toxin-resistant regenerated plants were male-fertile; the tassels of the remaining "male-sterile" plants did not resemble those on control *cms*-T plants. Toxin resistance and male fertility were transmitted to progeny of regenerated plants only through the female. Mitochondria isolated from two toxin-resistant cell lines were insensitive to the effects of the toxin. Based on previously characterized genetic and physiological differences between *cms*-T and N cytoplasms, it is not surprising that selection for toxin resistance yielded cytoplasmically controlled traits. This result indicates either that the contribution of nuclear genes to the cellular mechanism of toxin resistance is slight or that nuclear genes are subjected to less selection pressure than are extranuclear gene(s) by this selection method.

The mechanism by which these selected differences in the genetic capacity of the cytoplasm arose is not known. A possibility is that one locus of an extranuclear genome conditions both phenotypic alternatives: male sterility and susceptibility *vs.* male fertility and resistance. In this case, selection for a toxin-resistance mutation likely would be accompanied by a change to male fertility.

Rather than selection for a mutation, an alternative possibility is that the cytoplasmic genome of interest is heterogeneous, perhaps consisting of a mixture of *cms*-T and "normal" genomes. Selection against the *cms*-T genome would result in retention of the genome bearing the toxin-resistance locus. Male fertility would be selected concomitantly if only one locus was involved or if another locus on the same "normal" genome conditioned fertility and recombination did not occur. For this model of *cms*-T cytoplasm to give the correct plant phenotype, expression of the "normal" genome must be prevented, perhaps by a threshold effect based on relatively few "normal" genomes in the population or by a *trans*-dominant effect of the *cms*-T genome. Pollen transmission of extranuclear genomes is not a prerequisite for heterogeneity if one genome type was not exclusively selected during the evolution of *cms*-T cytoplasm.

The identity of the extranuclear genome is not known. Recent studies by Levings and Pring (15) have shown that restriction endonuclease digestion patterns of mitochondrial DNA were indistinguishable for five *cms*-T genotypes. However, patterns for the same genotypes with N cytoplasm were distinguishable from the *cms*-T mitochondrial DNA patterns obtained with the same restriction endonuclease. Similar analyses of chloroplast DNA from N and *cms*-T lines have not indicated restriction pattern differences (D. R. Pring and C. S. Levings III, personal communication). The analyses of or-

Table 4. Activity of mitochondria isolated from toxin-sensitive shoots and callus and toxin-resistant cell lines, with and without 2.5% toxin added to reaction mixtures*

Source†	NADH oxidation, nmol O_2/min · mg protein		DCPIP reduction,‡ ΔA_{520}/min · mg protein		ADP/O		Swelling, ΔA_{525}/mg protein	
	Control	Toxin	Control	Toxin	Control	Toxin	Control	Toxin
Shoots	26	50§	1.15	0.16§	1.14	0.0§	0	0.15§
Unselected callus	85	111§	0.72	0.22	0.75	0.0§	0	0.22§
R1 cell line	35	33	0.45	0.46	1.05	0.94	0	0
R2 cell line	40	37	0.50	0.50	0.86	0.86	0	0

* Reaction conditions were as described by Gengenbach and Green (7).
† Material derived from $BC_1A188(T)$. Shoots were obtained from germinating seeds; R1 and R2 were toxin-resistant cell lines.
‡ Dichlorophenolindophenol reduction.
§ $P \leq 0.01$ for difference between toxin and control.